Monster potential meets potential monster; the pros and cons of deploying genetically modified microalgae for biofuels production

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ABSTRACT

Biofuels production from microalgae attracts much attention but remains an unproven technology. We explore routes to enhance production through modifications to a range of generic microalgal physiological characteristics. Our analysis shows that biofuels production may be enhanced ca. 5 fold through genetic modification (GM) of factors affecting growth rate, respiration, photoacclimation, photosynthesis efficiency and the minimum cell quotas for nitrogen and phosphorous (N:C and P:C). However, simulations indicate that the ideal GM microalgae for commercial deployment could, on escape to the environment, become a harmful algal bloom species *par* excellence, with attendant risks to ecosystems and livelihoods. In large measure this is because an organism able to produce carbohydrate and/or lipid at high rates, providing stock metabolites for biofuels production, will also be able to attain a stoichiometric composition that will be far from optimal as food for the support of zooplankton growth. This composition could suppress or even halt the grazing activity that would otherwise control the microalgal growth in nature. In consequence we recommend that the genetic manipulation of microalgae, with inherent consequences on a scale comparable to geoengineering, should be considered under strict international regulation.

1. INTRODUCTION

The production of liquid transport biofuels from terrestrial crop plants is a proven technology (Smith *et al*. 2009) that continues to attract controversy. Much concern is levelled at the comparative societal, ethical and political values of using land and fertilizer for energy rather than feeding populaces. An alternative to the use of photosynthetic higher plants is to use photosynthetic microalgae. The term "microalgae" typically describes any photosynthetic microbe, either prokaryotic cyanobacteria or eukaryotic protists. While some of these organisms are capable of synthesising biochemical precursors for biofuels heterotrophically (Chen *et al*. 2011), net C-fixation requires a predominately photosynthetic metabolism under conditions of adequate illumination and (usually) inorganic nutrients. It is these photosynthetic microalgae that we consider here. Microalgae have been suggested to be ideal organism for biofuels production owing to their rapid growth rate, high oil content, suitability for growth on marginal land, and no direct conflict with the growth of food crops (Chisti 2007; Wijffels & Barbosa 2010). However, the path to successful deployment of microalgal biofuels is most challenging (Greenwell *et al.* 2010), with the cost estimates for production currently far exceeding fossil fuel prices (Williams & Laurens 2010; Shirvani *et al.* 2011).

Irrespective of the form of biofuels produced from microalgae, the objective is to transfer the normal flow of newly fixed carbon (C), from generating structural biomass, towards the accumulation of energy dense C storage products (starch, lipid). While nutrient (especially nitrogen, N) limitation prior to crop harvesting is needed to optimise biofuels production, light limitation is a far more likely event at this stage of microalgae crop growth owing to the self-shading properties of dense, highly pigmented, microalgal suspensions (Flynn *et al*. 2010). As with all commercial crops, approaches to overcoming such inherent limitations to production have attracted considerable interest (Beer *et al.* 2009; Li *et al.* 2010; Radakovits *et al*. 2010).

In this work we consider various issues associated with the advantages and disadvantages of applying genetic modification (GM) to microalgae to enhance biofuels production. Similar arguments to those we present here apply to any manipulation of the phenotypic characteristics of microalgae; we use the term genetic modification (GM) to imply any alteration of wild-type characteristics (e.g., Courchesne *et al*. 2009) that would not likely occur naturally. Since these organisms are single-celled microbes with minimum generation times of less than a day, they may be considered more readily amenable to GM than are higher plants. However, it is worth noting that in reality the path to GM of these organisms is far from trivial (Beer *et al*. 2009; Courchesne *et al*. 2009).

While deployment of GM biofuels-optimised microalgae may appear to offer great potential, there are counters to this promise. While microalgae in nature, as phytoplankton, are important components of the trophic web leading to fisheries, one may question whether microalgae optimised for biofuels production would readily fit benignly into ecology, or whether they may form harmful algal blooms (HABs). The large-scale growth of microorganisms that can be readily transferred across and between continents (e.g., with migrating wildfowl, or in the ballast water of ships (Hallegraeff 1998)) thus warrants careful consideration.

We have conducted our analysis through screening *in silico* GM algal populations, avoiding the attendant environmental and ethical risks of *in vivo* trials. *In silico* models of algal community physiology, though widely used in many oceanographic scenarios (e.g., Fasham *et al*. 2006), and deployed for simulations of microalgal biomass production (Flynn *et al*. 2010), have hitherto not been applied in earnest to examine algal biofuels production. Here, we use a variant of a well documented algal physiology model (Flynn 2001, 2008a; Flynn *et al*. 2010) to investigate options for enhancing microalgal biofuels production through GM routes. We then take the resultant biofuels-optimised GM organism and consider the implications for predator-prey interactions if such an organism escaped into the natural environment. The results indicate that the configuration of a biofuels-optimised organism also describes an organism that, on escape to the natural environment, has the potential to form harmful algae blooms (HABs) on a scale greater than do naturally occurring species.

2. METHODS

2.1 Base algal model

All models represent a compromise between complexity and computational load. The model used here is broadly typical of the more complex examples of mechanistic adaptive microalgal models. The model describing the growth of microalgae was developed from a long line of models (Flynn 2001, 2003). This model type has a firm basis in physiology, and has been well validated in its performance against data for various phytoplankton species, growing under different conditions and various combinations of light, N, P, Fe, and/or Si limitation (Flynn 2001; John & Flynn 2002; Flynn 2003; Fasham *et al.* 2006; Flynn 2008a, 2008b; Flynn *et al.* 2008). The implementation here included a description of the interactions between light, nitrogen (N) and phosphorus (P), with photoacclimation according to the "Flynn-Geider" configuration described in Flynn *et al*. (2001).

Algal-C was allocated to nitrogenous components (protein and nucleic acids), and non-nitrogenous structural components, with the balance as surplus C attributed to components for potential exploitation as biofuels. The simulated contribution to biofuels material is calculated by reference to the cellular C:N ratio, and the absolute minimum cellular C:N (CN_{min}), using the following equation.

$$Cex_C = \frac{CN_{cell} - CN_{\min}}{CN_{cell}} = \frac{CN_{cell} - (CN_{core} + Cstruc_N)}{CN_{cell}} \quad (1)$$

The value of CN_{min} can be determined experimentally from N-replete ammonium grown microalgae. It comprises two main components; the C:N of the core cellular nitrogenous components (CN_{core}) which is primarily protein and nucleic acids, and the non-nitrogenous structural material (primarily membranes and cell wall) which is described here as a C:N value referenced to the N in the core ($Cstruc_N$). The value of CN_{core} is estimated to have a value of ca. 3.2 gC $(gN)^{-1}$, from the C:N value of protein and nucleic acids, and the contribution that these two make to the whole cell (Geider & La Roche 2002). $Cstruc_N$ is given as CN_{min}-CN_{core}; the typical value of CN_{min} is around 4 (Geider & La Roche 2002; Flynn 2008a), yielding a value of this non-nitrogenous $Cstruc_N$ in nutrient-replete cells of 0.8 gC $(gN)^{-1}$. The biochemical fractionation between different carbohydrates, fatty acids and lipids within the surplus C (Cex_C) is not described further within the model because there are insufficient data as yet to support such a development. The fractionation does not affect the central conclusions of our analysis (considered further in Discussion).

2.2. non-GM and GM configurations

The microalgal model described interactions between light (including photoacclimation), nutrients (N and P) and growth. The base (non-GM) model was configured to represent a typical microalgae with respect to C:N:P:Chl (Flynn 2001, 2008), and produces maximum simulated areal production rates similar to peak values in nature (ca. 4 gC m^{-2} d^{-1}) (Sarmiento & Gruber 2006). The default values of constants used for the non-GM configuration, and the ranges explored for GM configurations, are given in table 1. These are all phenotypic features for which there are, likely, many genotypic regulators. For example, altering the photosystem antenna size (Melis 2009) affects phenotypic features of the initial slope of the photosynthesis-irradiance curve (α^{Chl}) and also, depending on how the cell responds by altering the number of photosynthesis reaction centres, potentially the maximum pigment content ($ChlC_{max}$). An explanation of the features considered is given below.

2.2.1 Maximum growth rate (μ_{max}). This sets the maximum possible growth rate under optimal conditions. The maximum growth rate attainable in simulations was less than μ_{max} because growth was simulated in a light-dark cycle (see below). Engineering factors affecting this feature may require a consideration of the source wild-type cell line, cell cycle controls, and limitations on respiratory functions affecting synthesis and cell maintenance (Flynn 2009).

2.2.2 Respiration rates (basal and metabolic respiration). Basal respiration (BasRes; described here as a proportion of μ_{max}) includes that associated with cell maintenance, while metabolic respiration (ProtRes; described here as C respired for the assimilation of N from intracellular ammonium into protein and nucleic acids) is associated with new net synthesis of structural components. Added to respiration is the cost of reducing nitrate to ammonium before assimilating nitrate-N (equivalent to 1.71 gC per g nitrate-N (Flynn & Hipkin 1999)). Engineering a decrease in respiratory costs may require a consideration of features such as protein turnover rates and functioning of key biochemical pathways.

2.2.3 $ChlC_{max}$. This sets the maximum pigment content, described here as chlorophyll per unit of cell-C. In crude terms this limits the "greenness" of the individual cell. With decreasing light, notably due to self-shading within the cell suspension, photoacclimation within the cell stimulates an increase in photopigment content, to capture more photons for the individual cell. Through natural selection the value of $ChlC_{max}$ is expected to become elevated (Flynn *et al*. 2010), attaining as much as 0.08 g Chla (g cell-C)$^{-1}$ (Anning *et al*. 2000). However, such elevated levels cause internal self-shading and critically also self-shading at the population level which decreases efficiency for photosynthesis, and hence decreases overall production. Total productivity is enhanced greatly if the level of photoacclimation (the value of $ChlC_{max}$) is limited, though this is unlikely to be a stable selective trait (Flynn *et al*. 2010). Engineering this feature may require a consideration of altering photosystem antenna size (Beckmann *et al*. 2009) and/or the number of photosynthetic reaction centres.

2.2.4 α^{Chl}. This phenotypic feature affects the overall efficiency of the light-chemistry conversion process, with units of gC (mol photon)$^{-1}$ × (m^2 g^{-1} Chl). The rate of photosynthesis is thus a function of the available light, the pigment content (ChlC; section 2.2.3) and the

value of α^{Chl}; for further information see Geider *et al*. (1998), Flynn *et al*. (2001) and Flynn (2001, 2003). Various factors affect the value of α^{Chl}, including the photochemistry within the Z-scheme, and the level of self-shading within the cell (MacIntyre *et al*. 2002). Internal self-shading is affected by the antenna size (Chl per reaction centre), overall Chl:C (affected by $ChlC_{max}$), and cell size. The fundamental basis of life on Earth is thought to have been fixed some 2Ga ago (Shi *et al*. 2005), with natural selection then optimising the packaging of these key biochemical processes. Accordingly, enhancing the efficiency of the basis of photochemistry, a fundamental feature of cell biochemistry, would literally be a real life-changing event.

2.2.5 Photoacclimation rate (M). This is described by parameter M in Flynn *et al.* (2001), and affects the rate at which pigment content is up-regulated with photoacclimation when the illumination falls (see above on $ChlC_{max}$). Here, the most important feature affected by this rate was the increase in Chl:C on entry into the dark phase of the diel light-dark cycle. Engineering this feature would require modifying the acclimation response rate to darkness and/or to C-limitation.

2.2.6 NC_0 and KQN. These parameters, respectively, describe the minimum cellular N:C (the subsistence quota for N (Flynn 2008a)) and the efficiency of N utilisation (specifically the efficiency of the action of biosynthetic pathways associated with N-compounds (Flynn 2008a, 2008b)). Lowering NC_0 would also, most likely, involve a decreased need for basal respiration (i.e., a lowering of protein turnover and damage-repair activities, with a decreased need for associated proteins/enzymes and RNA), and a lowering of the DNA content. Evidence from past experimental studies indicates that KQN sets a linear relationship between cellular N:C and growth rate (Flynn 2008a, 2008b). To decrease KQN, to make the relationship between N:C and growth rate curvi-linear, would require a fundamental change in protein and enzyme synthesis and efficiencies of their operation.

2.2.7 PC_0 and KQP. These are the P counterparts to NC_0 and KQN. There is far greater variability in these parameter values than for NC_0 and KQN, and they are recognised as important features in competition between microalgae (Lui *et al*, 2001; Flynn 2002). Because of the mixed structural and energetic/regulatory functions of P (contrasting with the mainly structural functions of N), the value of KQP is much lower than KQN, and the resultant often strongly curvi-linear relationship between P:C and growth rate indicates that the cells alter the

efficiency of P usage as P becomes limiting (Flynn 2008a, 2008b). To engineer changes in PC_0 and KQP would require decreasing the content of P-containing structural components (DNA, RNA, membranous phospholipids), and enhance the efficiency of use for the remainder.

2.2.8 CN_{core} and $Cstruc_N$. These parameters, respectively, describe the ratio by mass of the nitrogenous material in the cell (as C:N, comprising proteins, DNA and RNA) and the amount of organic non-nitrogenous structural material relative to the nitrogenous component (as C:N, comprising cell wall, membranes); see text associated with equation (1), above. To engineer changes in CN_{core} would require a decrease in the amount of DNA and RNA (as these contain a lower C:N than does protein (Geider & La Roche 2002)), if not a complete rebuild of the very nature of the biochemistry of life. Cell walls in microalgae are not as substantial as those in higher plants, so most of the material in $Cstruc_N$ comprises membranes containing phospholipids. Decreasing $Cstruc_N$ is thus likely to decrease PC_0. Default values used here are: CN_{core} = 3.2; $Cstruc_N$ = 0.8 (see Section 2.1).

2.3 Microalgal simulations

Growth was simulated with illumination conditions for a cloudless mid summer's day at latitude 0°. An astrological function was used describing the sigmoidal day-light variation with a noon maximum instantaneous photon flux density of 2180 µmol m^{-2} s^{-1} and, accounting for reflectance off the water surface with changing sunlight incidence, giving a day average of 675 µmol m^{-2} s^{-1}. It was assumed that conditions of temperature, CO_2 and pH were optimal throughout. The macro-nutrient regime was either that of f/2 (Guillard & Ryther 1962) with inorganic N available at 880µM and phosphate at 36.2µM, or at some multiple of those concentrations (e.g., 5 x f/2). Simulations to explore optimal configurations of growth and phenotype characteristics were run to steady-state in chemostat-style conditions, assuming a homogeneous distribution of cells over an optical depth of 0.1m, a depth shown previously to be in the optimal range to balance areal and volumetric production rates (Flynn *et al*. 2010). Physico-chemical limitations to the supply of nutrients (including CO_2 injection and the maintenance of pH) were assumed to have been overcome.

Areal production is reported for biomass and biofuels (with units of gC m^{-2} d^{-1}). As simulations were run in chemostat-style, steady-state mode, dilution rates in the plots equate to day-averaged growth rates. The biofuels component represents a portion of biomass, ranging typically from near zero to ca. 70% of the C-biomass, as according to equation 1.

2.4 Predator-prey simulations

For the predator-prey simulations, the base (non-GM) configured microalga or its GM-configured counterpart (table 2) were simulated as being grown together with a zooplankton predator. The zooplankton model (Mitra 2006) has been validated against various data sets, and used previously in the type of simulation deployed here (e.g., Mitra *et al*. 2007). The zooplankton parameters were set for a micro-zooplanktonic predator as per details in Mitra (2006); values for the maximum ingestion rate (G_{max}) and the half-saturation constant for ingestion (K_{pred}) are in table 2. The stoichiometric (C:N:P) basis of the trophic interactions described in the predator-prey simulations have a well known, firm, basis in the literature (Sterner & Elser 2002; Grover 2003; Mitra & Flynn 2005). In essence, an increasing disparity between the C:N:P of the microalgal prey and its zooplankton predator has a deleterious impact, adversely affecting growth of the predator and nutrient (ammonium and phosphate) regeneration.

Predator-prey simulations were run in a dynamic system describing a mixed layer depth of 10m, with mixing into and out of the mixed layer at 0.05 d^{-1}, and assuming an initial (and sub-mixed layer) nitrate concentration of 10µM. Phosphate was supplied at a mole ratio (nutrient N:P) of either 16 or 64, equating to pristine or eutrophically skewed conditions, respectively. Light at the surface was described as for the culture simulations (Section 2.3), however as the simulation developed the depth-integrated light field available to the microalgae decreased rapidly from an average daylight value of ca. 500 µmol photons m^{-2} s^{-1} at the start of the simulation to below 50 µmol photons m^{-2} s^{-1} at the peak of the bloom.

The predator-prey simulations presented here assume no genetic modification of algal fatty acid composition or of other factors that may adversely affect palatability to the predator (Mitra & Flynn 2005). As such, the results from the predator-prey simulations represent best-case scenarios. Genetic modification of the fatty acid content (profile) has already been explored (James *et al*. 2011; Radakovits *et al*. 2011; Lei *et al.* 2012), though the implications of this on palatability to grazers await clarification.

3. RESULTS

Additional results are presented in supplementary material available online (e-appendix) and are referenced as figure S1, S2 etc.

3.1 Optimization of biofuels production

The key results from our analyses of GM optimisation of production are summarised in figure 1; other results are given in the supplementary e-appendix (figures S1-S4). For optimising biofuels production, the most important phenotypic physiological features are maximising growth rate (μ_{max}; figure 1a), minimising the maximum photopigment content ($ChlC_{max}$; figure 1b), and maximising the efficiency of the light capture process (α^{Chl}; figure 1c). There are important, yet typically overlooked, differences between optimising production of microalgal biomass versus production of biofuels. Biofuels content (excess-C content) relates inversely to the N-limited status of the cells (equation 1), thus for maximum biofuels production (figure 1a) cells need to be grown under N-limiting conditions at their lowest relative growth rate (μ/μ_{max}). Therefore, although microalgae are typically grown commercially in systems operating at low dilution rates (and hence low μ), highest biofuels production will be realised through the use of cells with the highest potential for growth (high μ_{max}). Furthermore, avoiding light limitation is an essential prerequisite in the optimisation of biofuels production, thus limiting self-shading (by lowering $ChlC_{max}$) and maximising light-conversion to biomass (raising α^{Chl}) are critically important features. The form of the plots in figure 1a reflect this interplay between nutrient status, pigmentation and thus self shading, and production. Simulations using high nutrient loads (e.g., 5 x f/2) yielded low biofuels production, because the simulated organisms never exhausted the available nutrients (not shown).

Lowering the minimum cellular content of nitrogen (NC_0, figure 1d) and of phosphorus (PC_0, figure S1a), and enhancing the efficiency of the use of cellular N and P (KQP and KQN, figures S1b, S1c), give relatively minor headline enhancements of biofuels production, although there are additional advantages that would likely affect the financial viability of the whole venture (see Discussion). There are also several other physiological characteristics of lesser importance. Minimising respiration rates (figures S2a, S2b) prevents the loss of a proportion of the biofuels-C accumulated during day to support night-time respiration. Decreasing the rate of photoacclimation, the process by which microalgae increase their pigment content in response to light limitation (including at night time), is also useful (figure S2c), as it slows the self-shading event that decreases the accumulation of

290 excess-C. Lowering the C:N ratio of core nitrogenous components and of the amount of C
291 allocated to cell structure also have potential to slightly enhance biofuels production (figure
292 S3).
293 In reality, no GM changes will occur alone. In figure S4 we show the combined effects
294 of changing factors associated with photosynthesis, N or P physiology. Of these, the changed
295 photosynthetic configurations (figure S4a) are the most powerful, with scope even when used
296 alone to raise production by ca. 3 fold. While productivity gains through enhancing the
297 efficiency of the use of N and P (KQN and KQP) appear relatively minor, cost effectiveness
298 in fertilizer usage will improve.
299
300 **3.2 Predator-prey interactions**
301 Having explored the optimal configuration of microalgae for biofuels production, we
302 now consider whether zooplankton grazing could likely contain the escape of such an
303 organism to nature. Here, the growth conditions are very different, with a large optical depth
304 and low nutrient concentrations. We compared the predator-prey interactions between a
305 zooplanktonic predator (Mitra 2006) predating either a naturally configured (non-GM)
306 microalgal prey or a GM biofuels-optimised microalgal prey. For this, we used only a mid-
307 range GM configuration (table 2, Cf. table 1), but even this shows greatly improved biomass
308 and biofuels production capabilities over the non-GM form (figure S5).
309 Our simulations with the non-GM microalga show the expected importance of
310 elemental stoichiometry (C:N:P) in the predator-prey interaction (figure 2), with algal prey of
311 a high C content (high C:N and/or C:P) being of poor nutritional value. The adverse impact of
312 food quality on zooplankton becomes particularly apparent under P-limitation, i.e., under
313 nutrient-supply conditions with skewed N:P ratios typical of eutrophication (figure 2b). The
314 combination of characteristics in the GM biofuels-optimised microalgae gives a clear
315 enhanced potential for such organisms forming a poorly grazed bloom (figure 2). Firstly, this
316 status is attained through more rapid growth, forming higher population densities than given
317 by the comparative non-GM configuration for a given nutrient load, and thus out-stripping
318 zooplankton predation control. Secondly, there is the ability of the GM microalgae to become
319 more C-rich, exacerbating the already damaging skewed stoichiometry in nutrient-limited
320 microalgae, and hence disrupting the trophic dynamics which may otherwise restrain net
321 microalgal growth. Of the individual GM characteristics considered, those for μ_{max}, $ChlC_{max}$,
322 and especially PC_0 appear most important as potentially damaging characteristics in such an
323 organism released to nature (figures S6-S11). We also explored other combinations of

physical-nutrient descriptions (shallower versus deeper, with different nutrient loads), obtaining broadly similar responses, with the GM biofuels-optimised microalga always displaying an enhanced scope for forming large poorly grazed blooms (not shown).

4. DISCUSSION

4.1 The advantages of deployment of GM microalgae for biofuels production

Our analysis shows a potential for an increase in biofuels production from microalgae by perhaps five fold through modifying phenotypic characteristics. The exact gain will depend on many factors, but a gain of four fold is attainable by deploying the GM versus the non-GM configurations described in table 2, and these are not the extreme GM configurations tested (table 1). The optimal configuration for a biofuels producing microalgae is to have (in approximate order of importance) a high μ_{max}, high α^{Chl}, low $ChlC_{max}$, low minimum P:C and N:C contents, low photoacclimation and dark respiration rates, and high efficiency in the use of P and N. Collectively these features endow the organism with an ability to grow rapidly in low light conditions, use relatively little nutrients, more rapidly attain higher biomass and biofuels levels than normal, and be capable of attaining more extreme C:N and C:P levels, and hence contain more biofuels potential per unit of biomass. While such guidelines would help focus selection of wild-type algal strains, most likely a real enhancement would require specific attention to genetic modification of these phenotypic facets.

One feature, high maximum growth rates (μ_{max}), may appear surprising as a preferred characteristic given that continuous culture (chemostat-style) systems are typically run at low dilution rates, thus minimising consumption of fresh media. The reason for the importance of a high μ_{max} is because the production of excess C-rich metabolites that may act as stock for biofuels is driven primarily as a stress response to an excess supply of fixed C over supply of nutrients (notably of N). The greater the disparity between the growth rate (μ) and the potential maximum rate (μ_{max}), the greater the potential for the accumulation of excess-C; this is a function of the well documented relationship between cellular nutrient quotas and μ (Droop 1968, Flynn 2008). However, prolonged growth at low dilution rates (forcing low growth rates) selects for a decrease in μ_{max} (Droop 1974), presumably as the metabolism of the organisms downshifts through adaptation, thus minimising metabolic stress (Flynn 2009). This likely presents a challenge for the deployment of microalgae selected or genetically modified to achieve high growth rates, as with time the characteristic is likely to be lost (selected against) during growth at enforced low growth rates.

Lowering the minimum cellular content of nitrogen (NC_0, figure 1d) and phosphorus (PC_0, figure S1a), and enhancing the efficiency of the use of cellular N and P (KQP and KQN, figures S1b, S1c) appears to provide only minor enhancements of biofuels production. However, there are important operational and other commercial benefits of such configurations through minimising nutrient usage (Clarens *et al*. 2010; Greenwell *et al*. 2010). This is especially important for P as it is projected that readily available, relatively cheap, sources of phosphate will become increasingly limiting over the coming decades (Cordell *et al*. 2009). Additionally, the lower the P-demand by the microalgae the more likely it is that cells become N and not P limited when grown at a given nutrient N:P; this aids development of high C:N (Flynn 2008a, 2008b) and hence further enhances the potential for biofuels production.

Some of the features identified in our analysis would be easier to engineer than others and, critically, some will be more stable to mutation, selection and competition pressures. Already the photosystem antennae size has been subjected to GM (Beckmann *et al*. 2009; Melis 2009); this has some leverage on decreasing $ChlC_{max}$, and enhances production (figure 1b). While possession of a low $ChlC_{max}$ is likely not a stable trait (Flynn *et al*. 2010), minimising the use of nutrients is likely to be stable as it confers well documented advantages (Liu *et al*. 2001). Modifications of features influencing fundamental aspects of the generation of photoreductant, affecting the value of α^{Chl}, are likely to give stable traits though they will be far more challenging (perhaps currently impossible) to achieve.

A feature of the biofuels value of the microalgae that we do not explore in our analysis is the biochemical differentiation of the excess C between starch and lipid/ fatty acids. The nature of this surplus-C is important from a physiological perspective (as the synthesis of lipid is expensive relative to that of carbohydrate (Williams & Laurens 2010)), as well as from a biofuels perspective (Greenwell *et al*. 2010). While it is quite likely that attempts will continue to be made to genetically modify the biochemistry of this material (Beer *et al.* 2009; Li *et al*. 2010; Radakovits *et al.* 2010), and indeed other facets of the organism (such as features affecting the ease of harvesting, tolerance to temperature and salinity etc.), the characteristics that we consider represent primary features affecting microalgal growth and costs in terms of nutrient demand and production. The issue of such biochemical modification of lipid and/or carbohydrate does not affect the central message of the research presented here, not least because it does not alter the key features of the stoichiometric-inspired predator-prey interactions that we consider. Indeed, what will exacerbate the deterioration of the predator-prey interaction (increasingly the likelihood of a HAB) is a change in the quality

of the fatty acid such that it no longer contains metabolically important PUFA (Dalsgaard *et al*. 2003) and/or it contains a higher proportion of biofuels-desirable short chain saturate fatty acids (James *et al*. 2011; Radakovits *et al.* 2011), or even includes exotic fatty acids that are indigestible, unpalatable, or toxic.

4.2 Potential environmental risks posed by biofuels-optimized GM-microalgae

For microalgae to provide any significant contribution to biofuels production they will need to be grown over vast areas. It is most unlikely that all of that growth would be under cover, and even then it is unrealistic to expect that leakage or spillage of some proportion of the many thousands of cubic metres of culture that would be harvested per week would never occur. In all reality then, we need to consider the impact of such a leakage to the environment.

The features of a biofuels-optimised microalga, and their likely genetic stability, have important implications for ecology when such an organism enters the natural environment. Previously we reported that during algal-algal competition a microalga with a lower $ChlC_{max}$, while being superior as a clonal crop organism, would be at considerable selective disadvantage and would likely be eradicated in the natural environment (Flynn *et al*. 2010). However, as we warned in that previous work, this assumes that the control of growth by predators is equally distributed and does not discriminate in favour of the low $ChlC_{max}$ configured organism. Predator-prey systems are sensitive to such discriminations, and microalgae that appear outwardly poorly competitive with other microalgae can still grow to form dominant ungrazed blooms through such mechanisms (Mitra & Flynn 2006). The configuration of biofuels-optimised microalgae that we identify here is, other than the issue of $ChlC_{max}$, highly competitive in comparison with the default configuration. If such an organism became the dominant primary producer it would inevitably form dense blooms, for that is what it is designed for, albeit in ponds or other culture systems.

While the ability to grow rapidly under low light is important for competition with other phototrophs, of the factors we explored, it is the extreme C:N and C:P ratios in biofuels-optimized micoroalgae that create the greatest risk to trophic dynamics. Such extreme ratios, and the ability to continue to grow rapidly with high fatty acid and/or starch content creates a severe nutritional stoichiometric challenge for zooplankton growth (Grover 2003; Mitra & Flynn 2005, 2006; Mitra 2006). This limits predation upon the simulated GM organism, especially under P-limitation (figures 2b, S11).

While the potential formation of ungrazed HABs indicated in figure 2 simply reflects an imbalance in stoichiometric ecology, characteristics such as fatty acid (Dalsgaard *et al.*

2003) and toxin content (Mitra & Flynn 2006; Granéli & Flynn 2006) are of vital ecological importance, affecting zooplankton feeding and growth (Jones & Flynn 2005; Mitra & Flynn 2005). Any approach that alters fatty acid profiles in microalgae, especially to the biofuels-preferred shorter, saturated forms (James *et al.* 2011) which have little or no nutritional value to zooplankton, would undoubtedly exacerbate the significance of the already highly damaging stoichiometric imbalance (figure 2). Indeed, even when taken in isolation, modifying microalgae to alter their fatty acid content may be expected to adversely affect predation and increase the potential for them forming ungrazed (perhaps ungrazable) blooms.

The implications of changes in palatability and toxin production (as secondary metabolites in nutrient-stress microalgae), which are likely to co-occur with such fatty acid modifications, are well known (Jones & Flynn 2005; Mitra & Flynn 2005, 2006; Granéli & Flynn 2006). In consequence, it is most likely that biofuels-optimised microalgae will be less palatable than assumed in the simulations shown here, giving rise to what Mitra & Flynn (2005) refer to as negative stoichiometric modulation of predation (-ve SMP), a process that effectively shuts down predation very rapidly as C:N rises. The outcomes from such trophic interactions will thus likely be even starker in comparison with the default, wild-type, expectations.

One could endeavour to counter the above problems by developing traits that place biofuels microalgae at a distinct competitive disadvantage against their naturally occurring counterparts on escape to natural waters. However, configuring a crop organism in this way would also make it vulnerable to failure against contaminants in a culture system. The fact is that for a microalgae to be a robust commercially successful organism for biofuels production requires that it can outcompete any contaminating microalgae, and also proliferate in the presence of any zooplanktonic (predator) pests. Altering factors such as growth rate or nutrient affinity, so that GM microalgae would only grow well at high nutrient concentrations, would place them at a disadvantage in competition with contaminants in culture systems, and would in any case be selected against even within a clonal crop culture when growing under the nutrient limitation that is required to stimulate biofuels production.

One potential solution to this conundrum is to optimise growth of GM biofuels-optimised microalgae in extreme environments, for example with respect to temperature or pH (Spijkerman & Wacker 2011), conditions that would not commonly occur in nature. Whether such growth conditions place an acceptable additional financial and logistic burden on the whole enterprise would need careful consideration, given the massive volumetric scale

458 of biomass production required to provide a significant biofuels production. Such an approach
459 would also itself not be immune from posing risks to the environment.
460 An alternative approach is not to increase biomass production to yield metabolites for
461 biofuels production, but to modify biochemistry to redirect the synthesis of organics away
462 from growth and towards fatty acids, which the cells then release for direct harvesting from
463 the growth medium (Liu *et al*. 2011). This approach could be viewed as having parallels with
464 events that already occur in nature. The production and release of excess polysaccharide from
465 nutrient-stressed microalgae in nature is not uncommon, and causes well documented
466 problems associated with foams and transparent exopolymeric material (Myklestad 1995;
467 Schilling & Zessner 2011). This released material then promotes the growth of ecosystem
468 disruptive algal blooms through inhibition of grazing, and can also create serious pollution
469 events along coasts (Seuront *et al*., 2006). While the GM approach to direct extracellular
470 production of material destined for biofuels carries various attraction (notably with respect to
471 harvesting), it may thus also carry with it causes of environmental concern as well.
472 Immobilising the microalgae on some fixed substrate could overcome the risk, assuming that
473 the cells could only grow on the substrate and that challenges of adequate illumination (and
474 hence production) can be overcome amongst the attached microalgae.
475 Finally, it is worth noting that GM terrestrial crops differ greatly from GM microalgae
476 with respect to the potential for environmental damage. While higher plants can be made
477 sterile to limit their spread, by their very nature GM microalgae must be capable of
478 reproduction. Higher plants undergo typically one generation a year; microalgae reproduce
479 daily. Our understanding of the impacts of GM higher plants upon ecology has developed
480 over a few decades, a period of reproductive cycles that GM microalgae would achieve in a
481 week. It will thus take something of the order of a century of higher plant generations to
482 compare with a fraction of one year's growth of microalgal generations. While GM terrestrial
483 plant crops have been deployed without obvious catastrophic impacts on ecology (though
484 certainly not without controversy on this point; Tilman *et al*. 2009), it is not possible to
485 extrapolate an argument that GM microalgae would be similarly benign.
486
487 **5. CONCLUSIONS**
488 There has been much claimed for the potential of algal biofuels to contribute
489 significantly to energy sustainability and security, but detailed analyses indicate that for
490 financial and logistic realisation costs per litre of biofuels need to come down significantly
491 before such a dream can be realised (Clarens *et al.* 2010; Greenwell *et al.* 2010; Williams &

492 Laurens 2010). A major advance may be achieved by attaining a step change in microalgal
493 productivity. Significantly, our previous analysis (Flynn *et al.* 2010) suggests that areal
494 productivity using "typical" microalgae is likely to be little better than that seen under optimal
495 conditions in nature (Sarmiento & Gruber 2006). While in culture ponds the volumetric
496 production is much higher, and hence harvesting and dewatering costs are decreased
497 accordingly, the implication is that areal production using wild-type strains is limited by the
498 total light incident to the culture system and by the underlying physiology of the organisms.
499 That physiology has evolved over millions of years from basic metabolic building blocks with
500 origins to the emergence of life on Earth (Shi *et al*. 2005). To go beyond this (natural
501 maximum) productivity of ca. 4 gC m^{-2} d^{-1} thus requires a change in the physiology of the
502 organisms. It is most likely that this can only be achieved through radical genetic
503 modification, creating organisms that are literally new to nature.
504 Our work indicates a clear potential for GM in the commercial development of
505 microalgal biofuels, with scope for raising production by perhaps half an order of magnitude
506 (figures 1, S5). Coupled with more efficient processing technologies, GM microalgae could
507 make microalgal biofuels a viable and cost-effective option. However, our study also suggests
508 a very real risk that the engineered product could come to represent the perfect harmful algal
509 bloom (HAB) species (figure 2), with all the attendant risks to the environment, to
510 environmental services and human health that HABs present (Glibert *et al*. 2005). This is not
511 to say that all GM approaches will exhibit the same potential risks to nature. However, and
512 accepting that not all of the GM traits may be stable in nature, given the ease with which GM
513 microalgae could be transferred around the planet the potential risk of GM microalgae to
514 nature should not be underestimated. There already exists ample warning of the damage that
515 can be caused from the inadvertent trans-ocean transfers of "exotic" natural HAB species
516 (Hallegraeff 1998), with no evidence that naturally occurring zooplankton can contain the
517 problem. Indeed, disruption to biodiversity by invasive alien species is well known and all too
518 common (e.g., for aquatics, Padilla & Williams 2004). In this capacity, the mass cultivation of
519 any microalga isolated from a source distant to the site of commercial deployment is also a
520 matter of concern.
521 The spread of a GM-microalgae of the type of configuration we identify would be
522 effectively impossible to halt. As GM of factors likely affecting palatability of microalgae is
523 already being conducted in the name of biofuels production (Li *et al, 2010;* Radakovits *et al.*
524 2011; Lei *et al.* 2012), there is a real risk that the genie is already part way out of the bottle. If
525 GM biofuels-optimised microalgae were to destroy fisheries then a main driver for microalgal

biofuels research, the argument that such biofuels production would not compete with production of biomass for food (Chisti 2007; Wijffels & Barbosa 2010), may prove to be totally misplaced. Accordingly, a strong argument can be made for the regulation of GM of microalgal at an international level, because the potential for damage could have global consequences, echoing recent concerns over geoengineering (McNaughton & Owens 2012). Whether, against arguments for sovereign fuel security, regulation could be enforced, is a dilemma that society may soon have to face up to.

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LEGENDS

Table 1

Parameter values for the base non-GM model and ranges explored for the GM counterparts. Values are also given for the physico-chemical culture system; also see text. The nutrient regime equates to that of the classic f/2 medium (Guillard & Ryther 1962) containing 882µM N and 36.2µM P

Table 2

Parameters for model runs shown in figure 2 and as indicated in figure legends for figures S5-S11. See Methods, and table 1 for further information.

Figure 1. Biomass and biofuels areal production. Variation of areal production of biomass (left hand plots) and biofuels (middle plots) against dilution rate (=growth rate, µ) for different physiological characteristics under chemostat-style steady-state conditions. The right hand plots show the percentage of biomass as biofuels; note the different axes directions. Part (a), maximum growth rate (μ_{max}). Part (b), maximum chlorophyll content ($ChlC_{max}$). Part (c), overall phenotypic efficiency of the photochemistry (α^{Chl}, given here with units of (mgC µmol^{-1} photon) (m^{2}g^{-1} chl.a)). Part (d), minimum (subsistence) quota for N (NC_0, N:C). Note that at high dilution rates biofuels production falls as the microalgae become N-sufficient.

Figure 2. Predator-prey simulations. Simulated interaction between a microalgal prey (Algae) and its zooplanktonic predator (Zoo). Simulations were run with the microalga configured to represent a non-GM (thin line) or a biofuels optimised GM strain (thick line); see table 2. In panel (a) the mole nutrient ratio is N:P = 16 representing pristine water bodies. In panel (b) N:P = 64, representing the skewed nutrient content seen in eutrophic coastal waters. Temporal development of the interaction would depend on initial conditions. Plots show development of the algal and zooplankton biomass, and changes in the algal N:C and P:C ratios. Decreases in algal N:C and P:C indicate changes in nutrient stress (through exhaustion of external nutrient supply).

SUPPLEMENTARY FIGURES

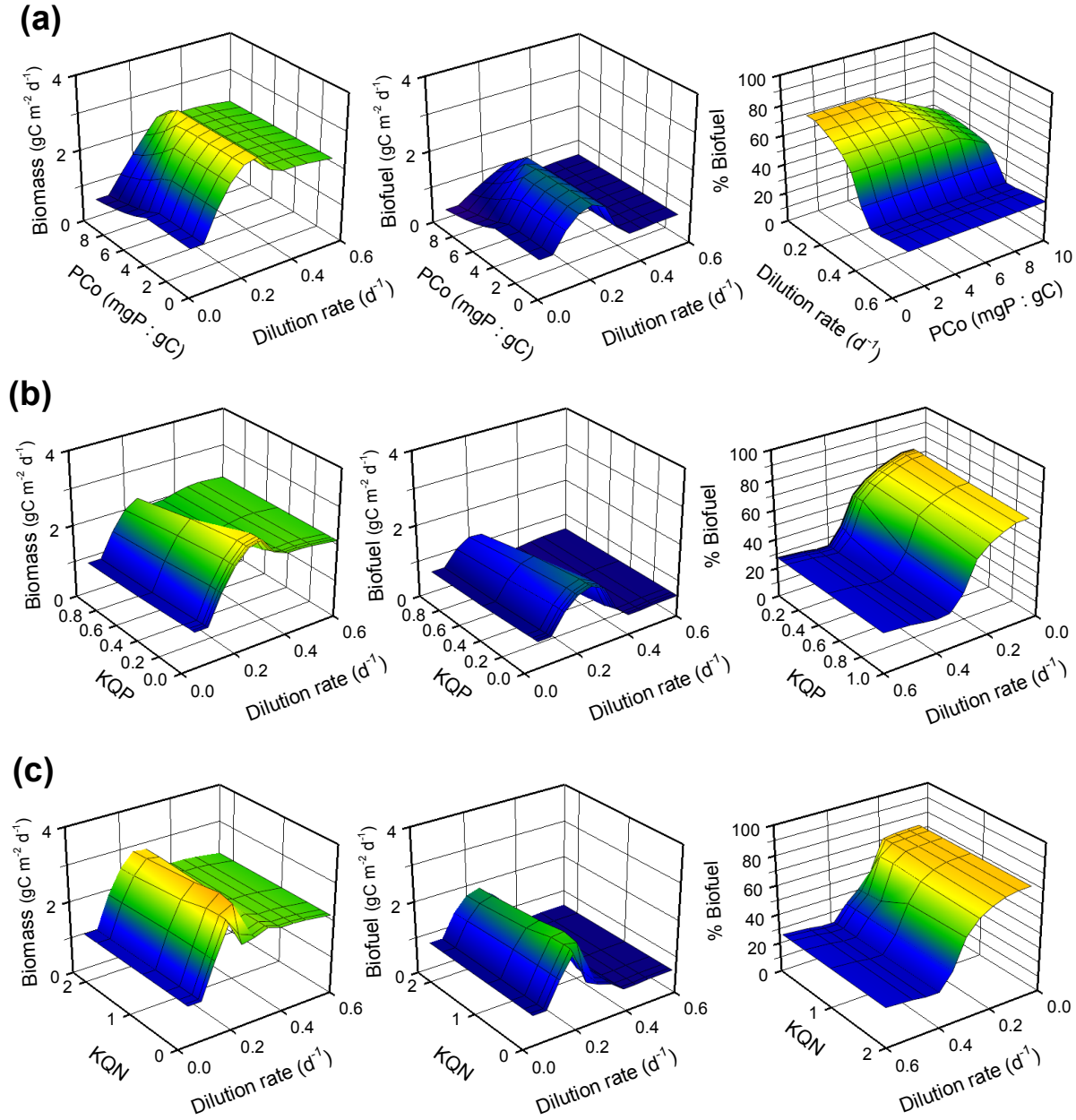

Figure S1. Variation of biomass (left hand plots) and biofuels (middle plots) areal production against dilution rate (=growth rate, μ) for different physiological characteristics. The right hand plots show the percentage of biomass as biofuels; note the different axes directions. Part (a), the minimum (subsistence) quota for P (PC_0); see figure1d for the N subsistence quota. Part (b), constant describing the efficiency of P utilisation during growth (KQP, low values are more efficient). Part (c), constant describing the efficiency of N utilisation during growth (KQN, low values are more efficient). Note that at high dilution rates, biofuels production can fall as the microalgae become N-sufficient.

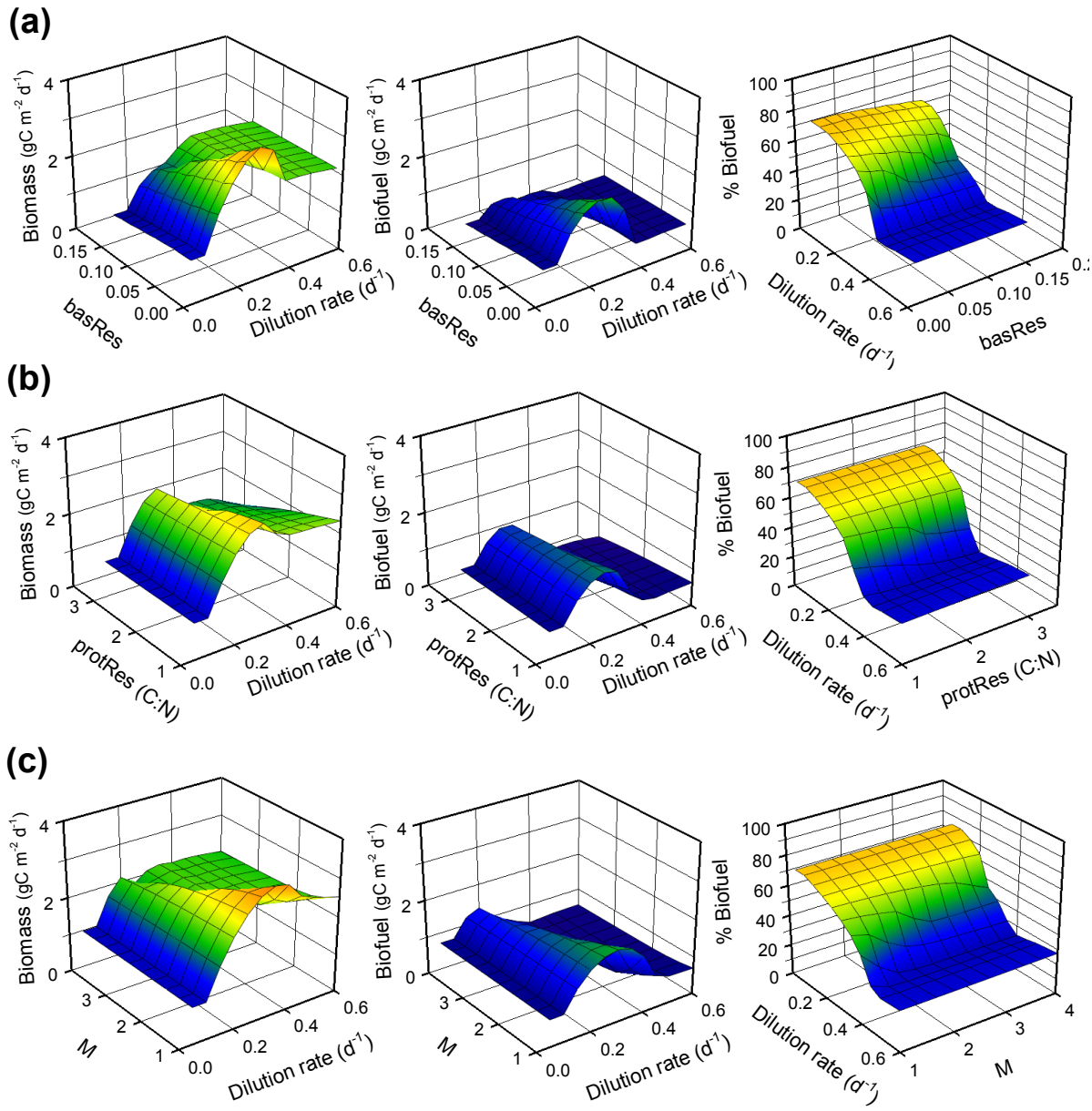

Figure S2. Variation of biomass (left hand plots) and biofuels (middle plots) areal production against dilution rate (=growth rate, μ) for different physiological characteristics. The right hand plots show the percentage of biomass as biofuels; note the different axes directions. Part (a), basal respiration rate (basRes, as a percentage of maximum growth rate). Part (b), metabolic respiration rate for protein synthesis (protRes). Part (c), index for the rate of photoacclimation (M). Note that at high dilution rates biofuels production can fall as the microalgae become N-sufficient.

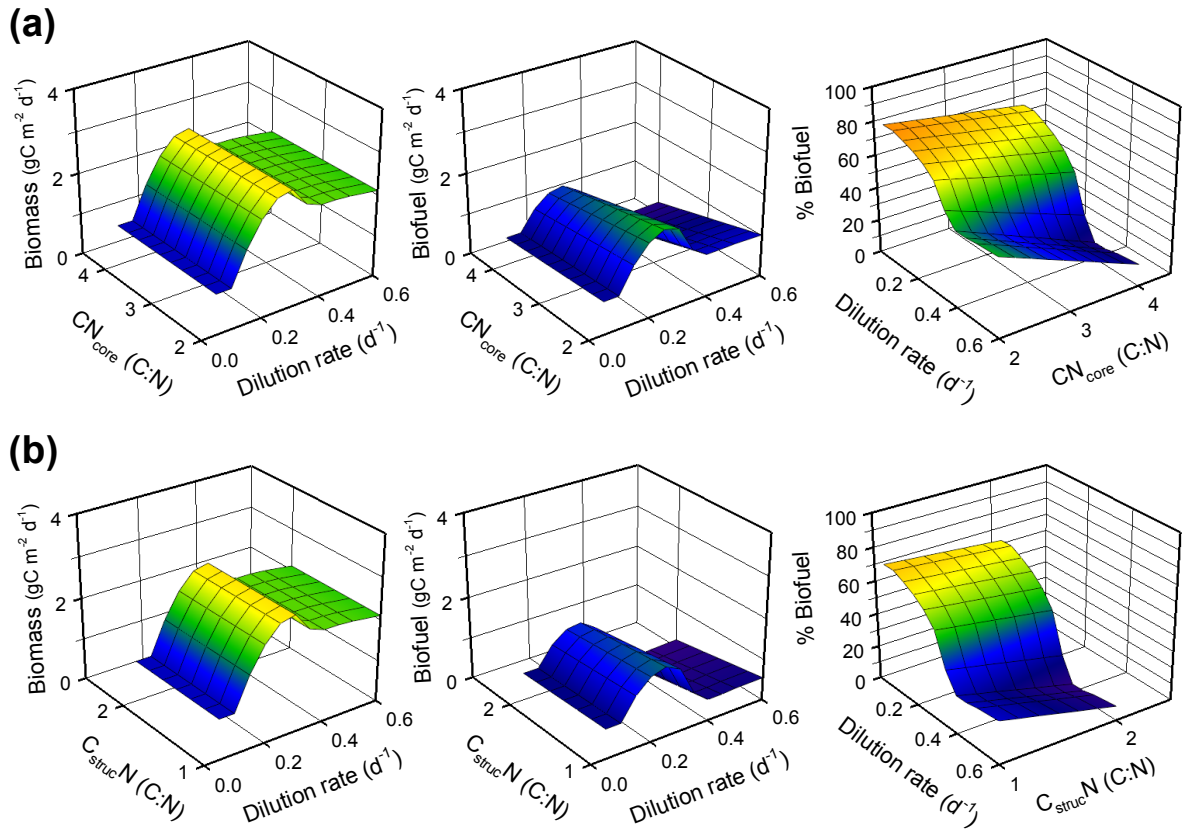

Figure S3. Variation of biomass (left hand plots) and biofuels (middle plots) areal production against dilution rate (=growth rate, μ) for different physiological characteristics. The right hand plots show the percentage of biomass as biofuels; note the different axes directions. Part (a), the C:N ratio for the core nitrogenous material (CN_{core}; protein + DNA + RNA). Part (b), the C allocation to the non-nitrogenous structural material (cell walls, membranes) as a ratio of the nitrogenous components ($C_{struc}N$). Note that at high dilution rates biofuels production can fall as the microalgae become N-sufficient.

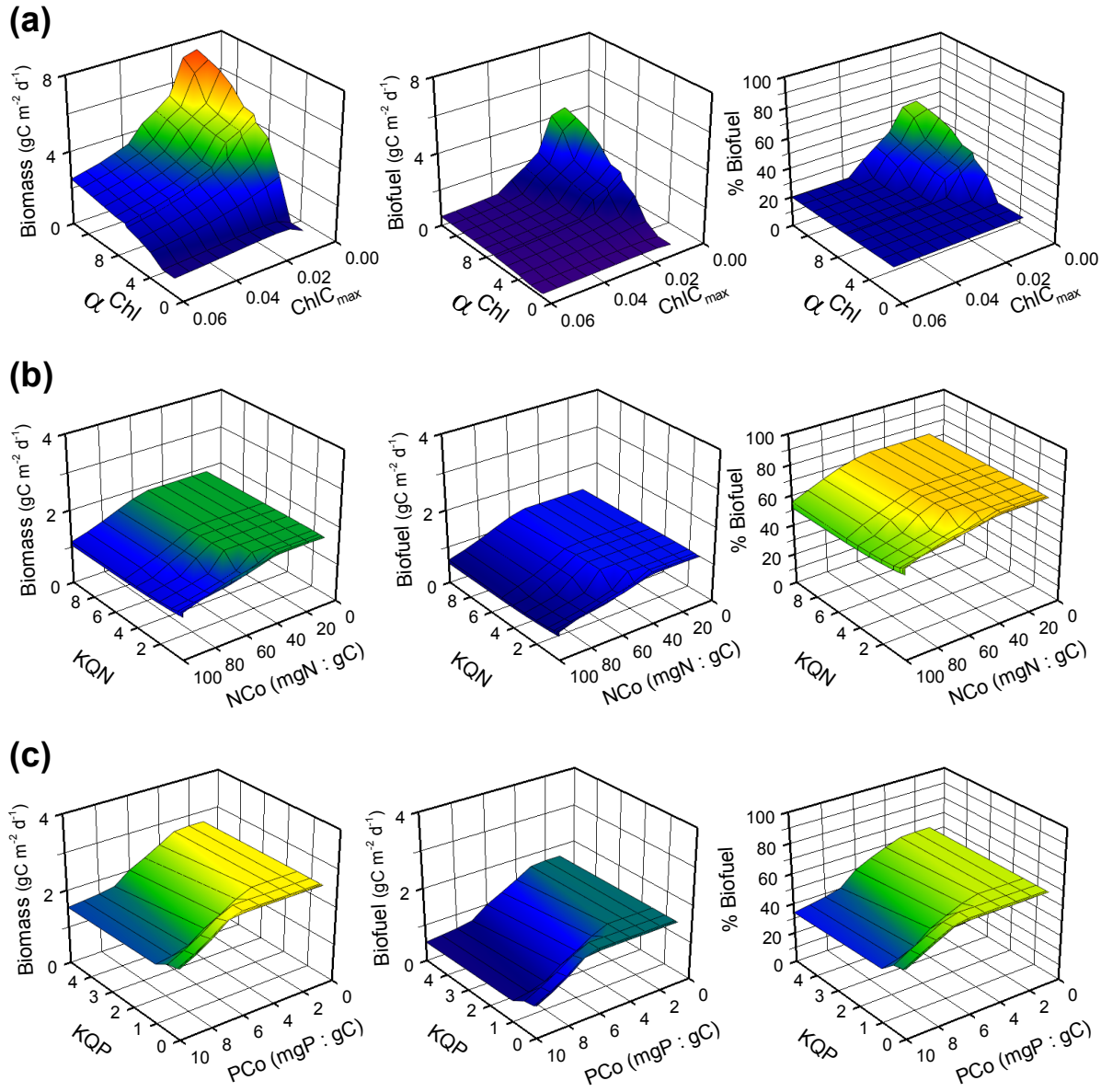

Figure S4. Variation of biomass (left hand plots) and biofuels (middle plots) areal production as a function of different pairs of physiological characteristics. The right hand plots show the percentage of biomass as biofuels. The dilution rate (= growth rate, μ) was fixed at 0.2 d^{-1}. Part (a), maximum Chl:C ($ChlC_{max}$) versus the overall phenotypic efficiency of the photochemistry (α^{Chl}, given here with units of (mgC μmol^{-1} photon) · ($m^2 g^{-1}$ chl.a)). Part (b), minimum (subsistence) quota for N (NC_0) versus the constant parameter describing efficiency of N utilisation during growth (KQN, low values are more efficient). Part (c), minimum (subsistence) quota for P (PC_0) versus the constant describing the efficiency of N utilisation during growth (KQP, low values are more efficient).

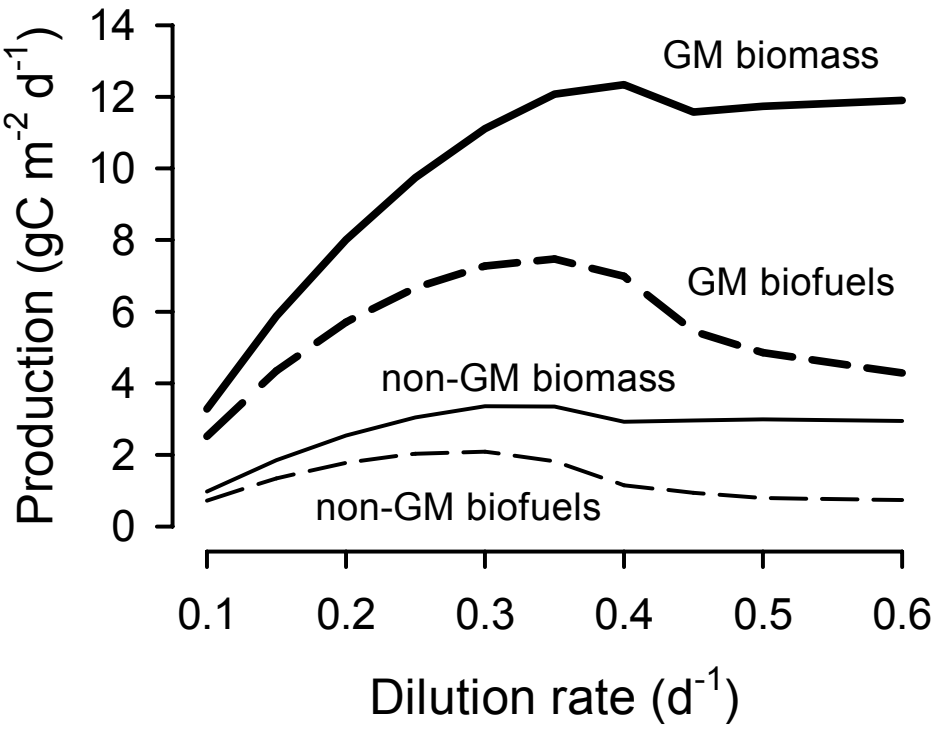

Figure S5. Steady-state rates of production using microalgae configured as non-GM or GM configurations as described in table 2. Maximum rates of production were achieved with nutrient concentrations of f/2 for the non-GM algae, but with 3 x f/2 for the GM algae; the latter configuration enables growth to higher densities because the pigmentation level is restricted (i.e., $ChlC_{max}$ is lower).

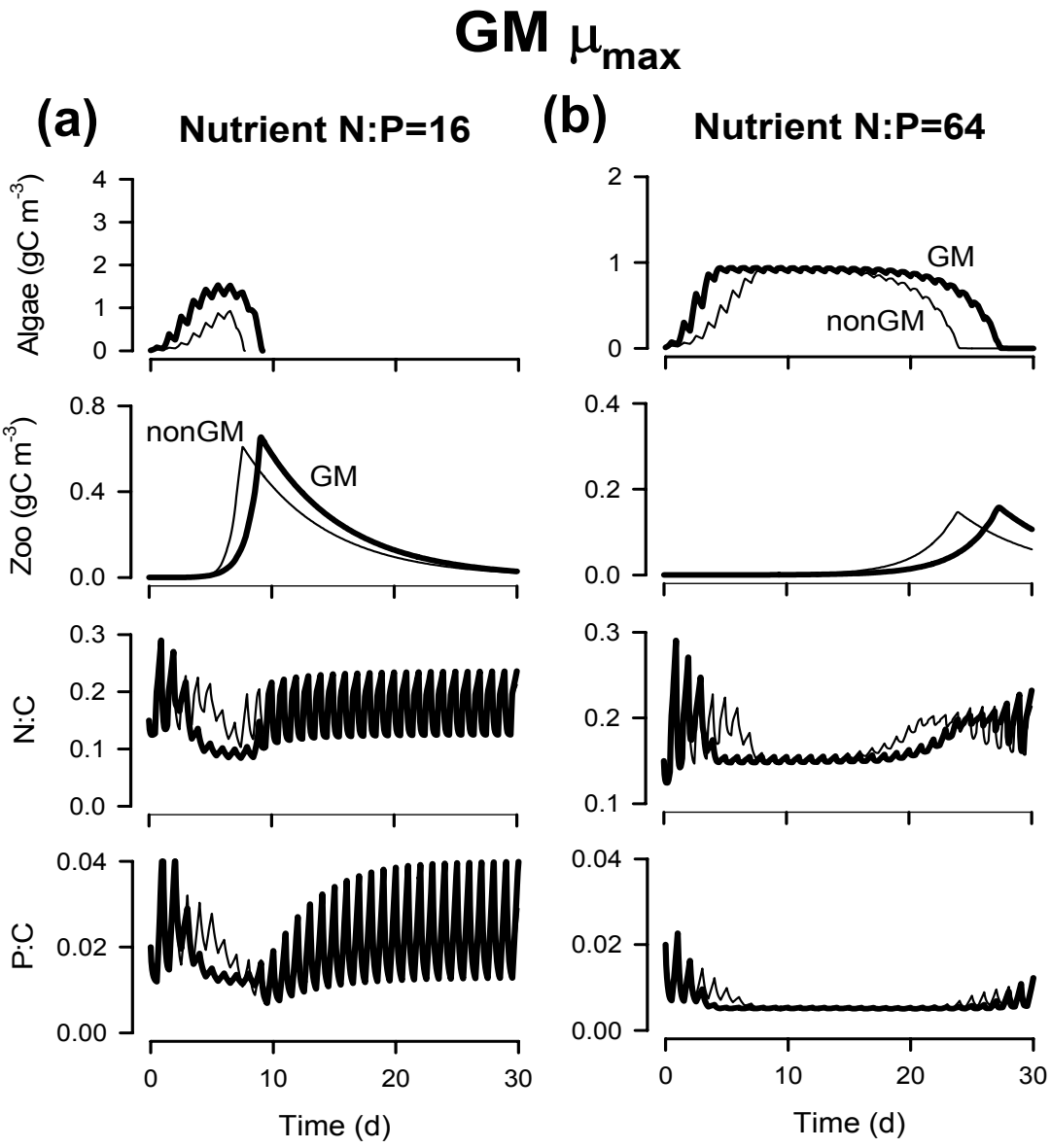

Figure S6. Simulated interaction between a microalga and its zooplanktonic predator. Simulations were run with the microalga configured to represent a non-GM or a GM strain with elevated μ_{max}. See table 2 for further details. In panel (a) the mole nutrient ratio is N:P 16, as expected in pristine water bodies. In panel (b) N:P is 64, representing the skewed nutrient content seen in eutrophic coastal waters. The temporal development of the interaction would depend on initial conditions. Plots show development of the algal and zooplankton biomass, and changes in the algal N:C and P:C ratios. Decreases in N:C and P:C indicate changes in nutrient stress (through exhaustion of external nutrient supply).

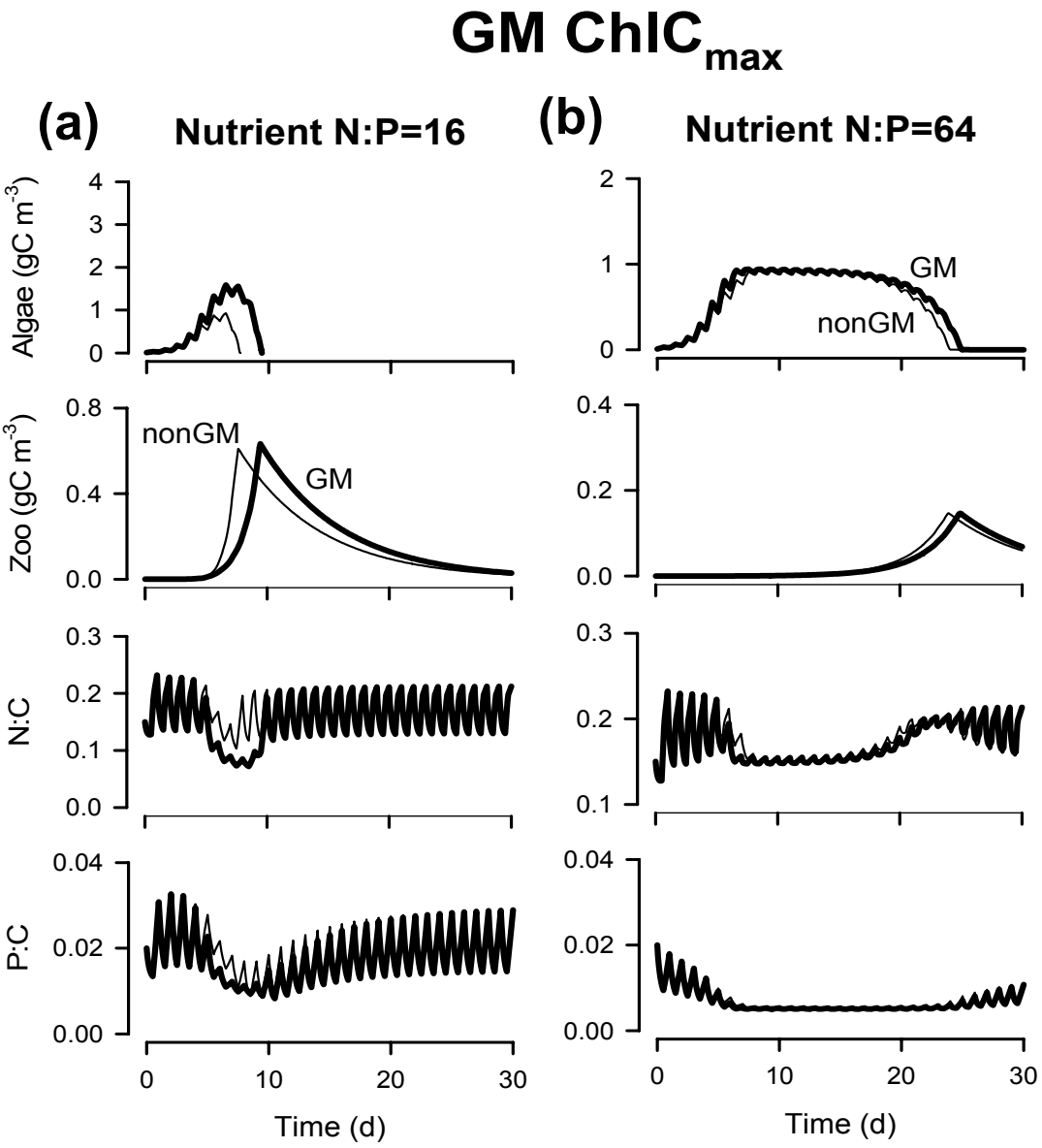

Figure S7. Simulated interaction between a microalga and its zooplanktonic predator. Simulations were run with the microalga configured to represent a non-GM or a GM strain with a depressed $ChlC_{max}$. See table 2 for further details. In panel (a) the mole nutrient ratio is N:P 16, as expected in pristine water bodies. In panel (b) N:P is 64, representing the skewed nutrient content seen in eutrophic coastal waters. The temporal development of the interaction would depend on initial conditions. Plots show development of the algal and zooplankton biomass, and changes in the algal N:C and P:C ratios. Decreases in N:C and P:C indicate changes in nutrient stress (through exhaustion of external nutrient supply).

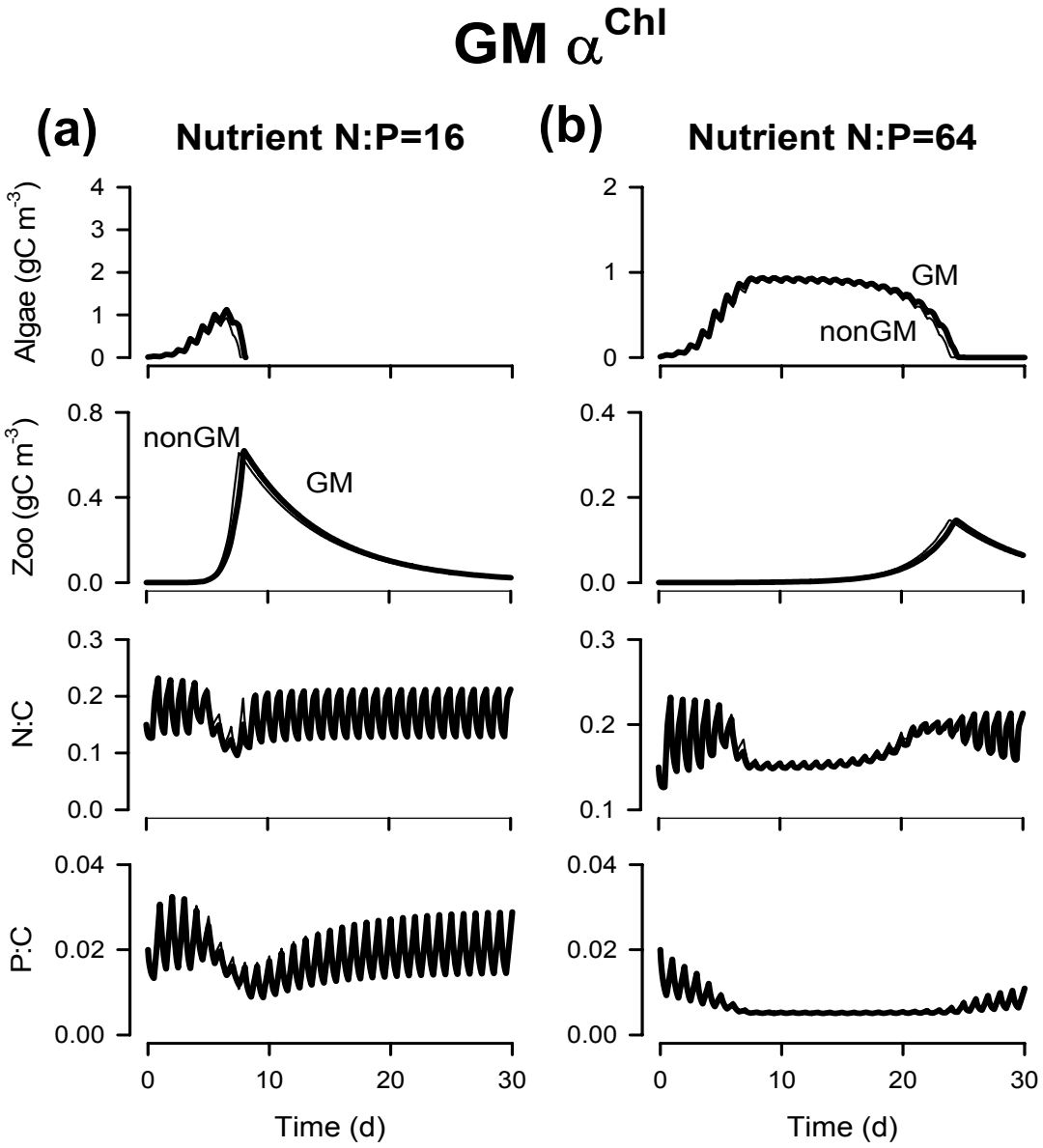

Figure S8. Simulated interaction between a microalga and its zooplanktonic predator. Simulations were run with the microalga configured to represent a non-GM or a GM strain with elevated α^{Chl}. See table 2 for further details. In panel (a) the mole nutrient ratio is N:P 16, as expected in pristine water bodies. In panel (b) N:P is 64, representing the skewed nutrient content seen in eutrophic coastal waters. The temporal development of the interaction would depend on initial conditions. Plots show development of the algal and zooplankton biomass, and changes in the algal N:C and P:C ratios. There are very few points of difference between the non-GM and GM configurations. Decreases in N:C and P:C indicate changes in nutrient stress (through exhaustion of external nutrient supply).

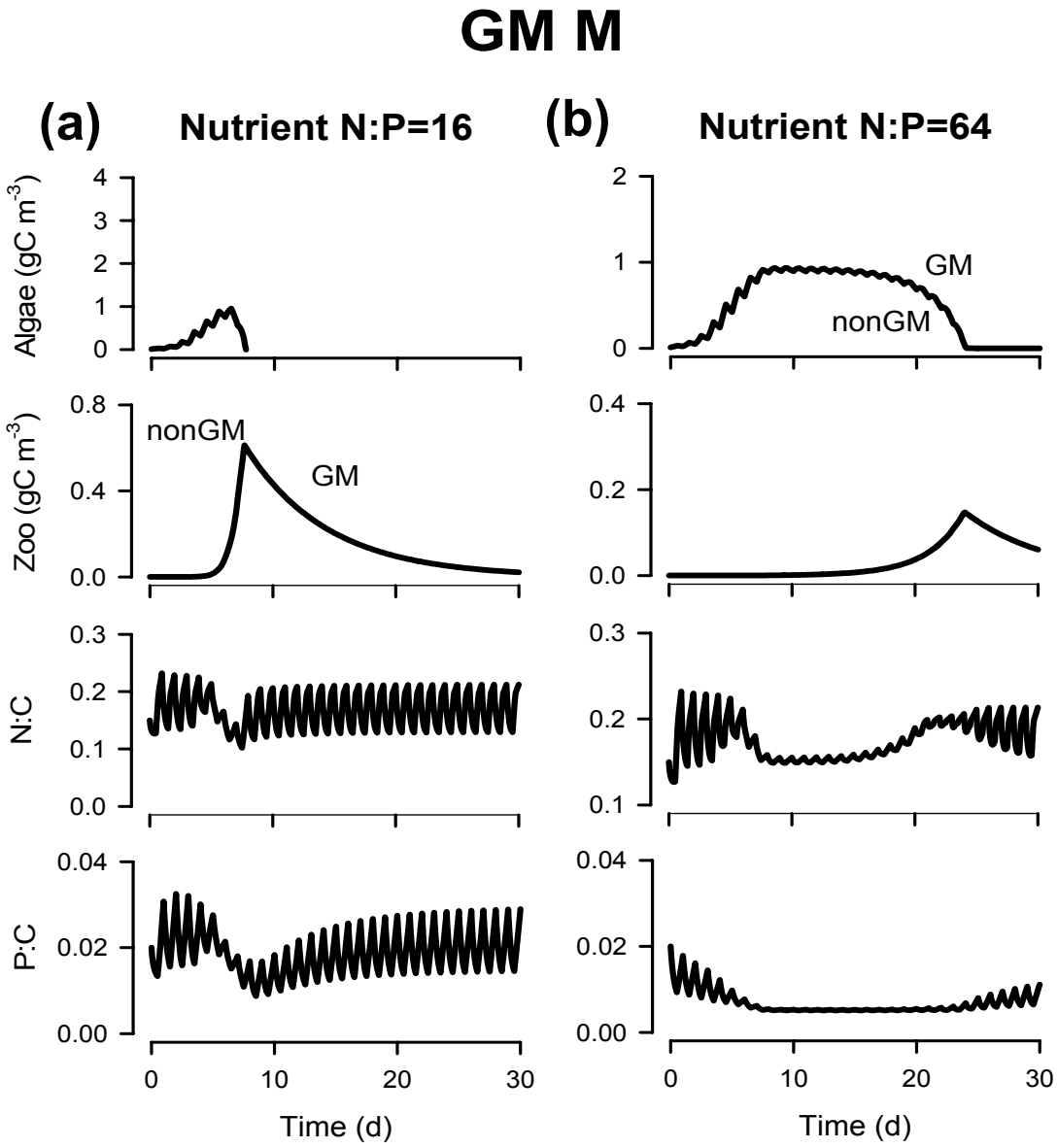

Figure S9. Simulated interaction between a microalga and its zooplanktonic predator. Simulations were run with the microalga configured to represent a non-GM or a GM strain with decreased M. See table 2 for further details. In panel (a) the mole nutrient ratio is N:P 16, as expected in pristine water bodies. In panel (b) N:P is 64, representing the skewed nutrient content seen in eutrophic coastal waters. The temporal development of the interaction would depend on initial conditions. Plots show development of the algal and zooplankton biomass, and changes in the algal N:C and P:C ratios. Decreases in N:C and P:C indicate changes in nutrient stress (through exhaustion of external nutrient supply). There are essentially no points of difference between the non-GM and GM configurations.

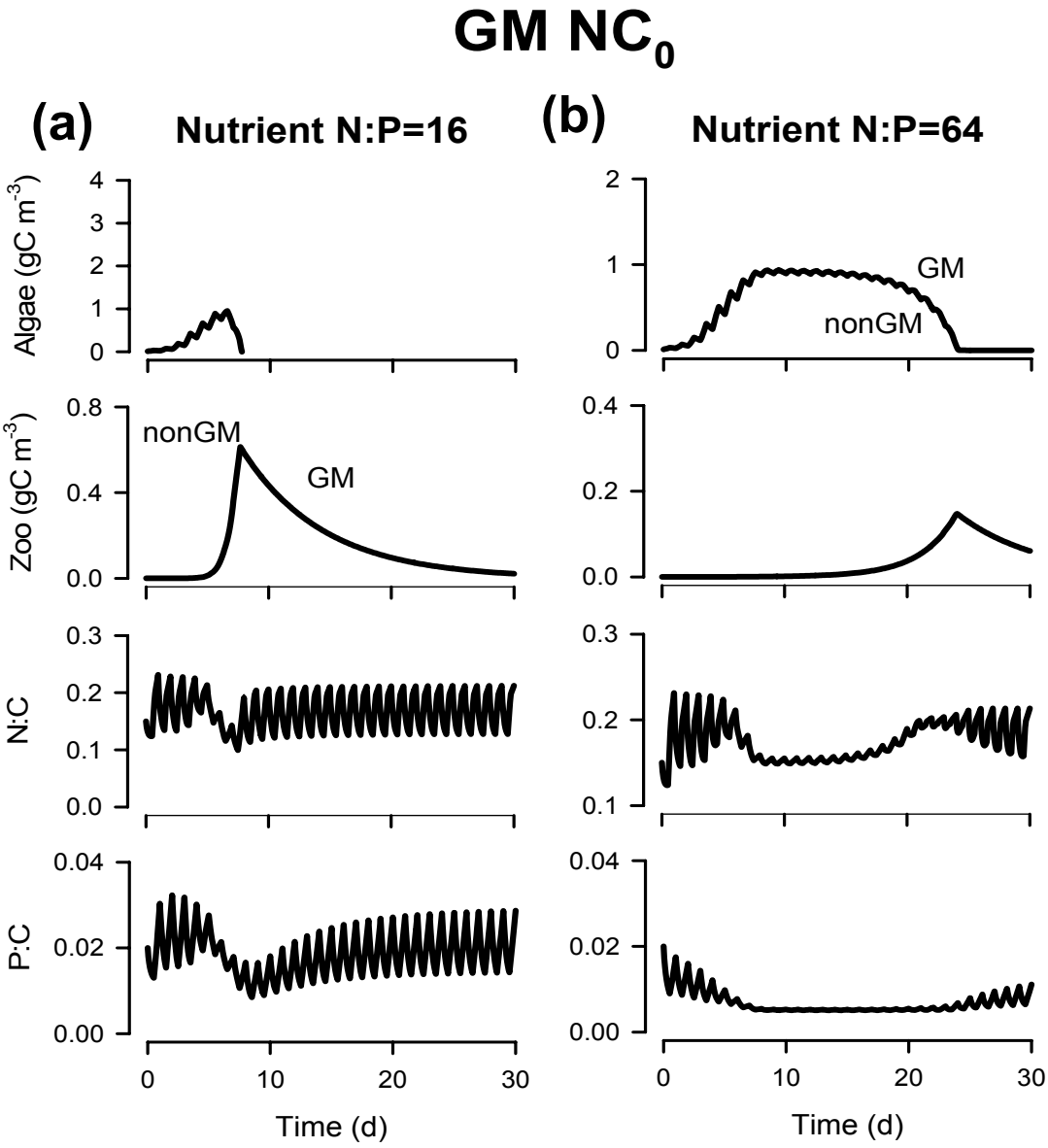

Figure S10. Simulated interaction between a microalga and its zooplanktonic predator. Simulations were run with the microalga configured to represent a non-GM or a GM strain with decreased NC_0. See table 2 for further details. In panel (a) the mole nutrient ratio is N:P 16, as expected in pristine water bodies. In panel (b) N:P is 64, representing the skewed nutrient content seen in eutrophic coastal waters. The temporal development of the interaction would depend on initial conditions. Plots show development of the algal and zooplankton biomass, and changes in the algal N:C and P:C ratios. Decreases in N:C and P:C indicate changes in nutrient stress (through exhaustion of external nutrient supply). There are essentially no points of difference between the non-GM and GM configurations.

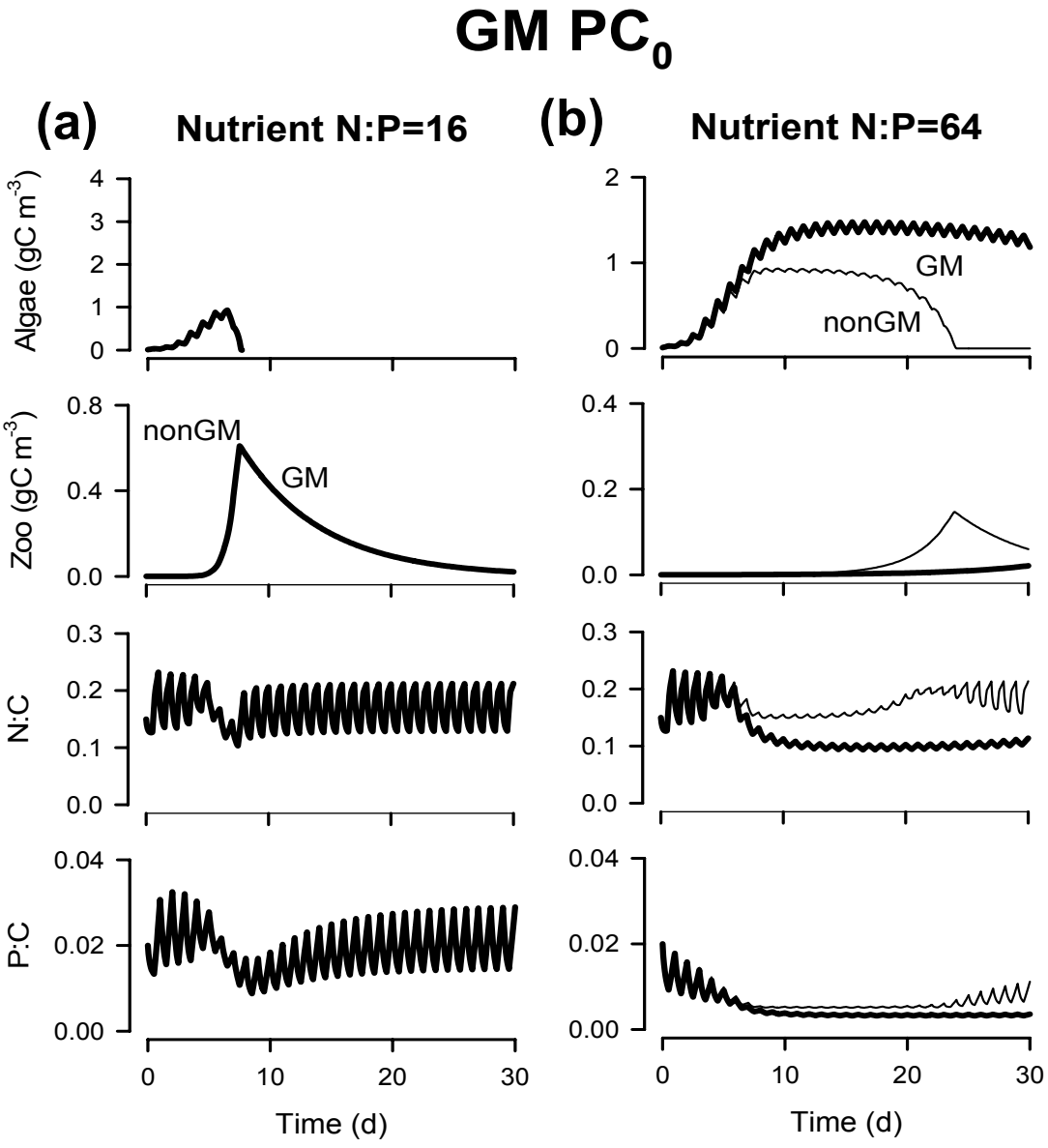

Figure S11. Simulated interaction between a microalga and its zooplanktonic predator. Simulations were run with the microalga configured to represent a non-GM or a GM strain with decreased PC_0. See table 2 for further details. In panel (a) the mole nutrient ratio is N:P 16, as expected in pristine water bodies. In panel (b) N:P is 64, representing the skewed nutrient content seen in eutrophic coastal waters. The temporal development of the interaction would depend on initial conditions. Plots show development of the algal and zooplankton biomass, and changes in the algal N:C and P:C ratios. Decreases in N:C and P:C indicate changes in nutrient stress (through exhaustion of external nutrient supply).